Bioluminescence ATP Assay for Estimating Total Plate Counts of Surface Microflora of Whole Cantaloupe and Determining Efficacy of Washing Treatments†

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ABSTRACT

The surface microflora of cantaloupes were estimated using a bioluminescence ATP assay, and results were compared to plate count data. Cantaloupes were treated as follows: (i) water washed, or (ii) washed in solutions of sodium hypochlorite (1,000 mg/liter) or hydrogen peroxide (5%) for 5 min. Bioluminescence ATP assay results showed differences in ATP level/cm^2 of cantaloupes dipped in chlorine or hydrogen peroxide solution; ATP levels in these washed samples were lower than in controls due to antimicrobial action of the treatments on the cantaloupe surface. Linear correlations were found between the bioluminescence ATP assay and aerobic plate counts of unwashed cantaloupe (r^2 = 0.995) and those washed with water (r^2 = 0.990) determined before storage. Lower correlations between the bioluminescence ATP assay and the aerobic plate counts were observed on cantaloupes stored for 120 h at 20°C (r^2 = 0.751) than at 4°C (r^2 = 0.980) without washing treatment. Lower correlation at 20°C may be the result of clusters or growth that occurred in chains. ATP levels of washed cantaloupes correlated well with bacterial plate counts (r^2 = 0.999). A reliable minimum detectable threshold using the bioluminescence ATP assay was established at 3 $\log_{10}$ fg/cm^2 corresponding to 4 $\log_{10}$ CFU/cm^2. Bioluminescence ATP assay is not recommended for washed samples where the microbial load is near or below the threshold. Therefore, the bioluminescence ATP assay will be recommended for quick estimation of total microbial load on cantaloupe surfaces where the population is expected to exceed this threshold. The assay can save the industry time by eliminating the required incubation required by the conventional methods.

The microflora of all food items are of practical significance to producers *(1, 4, 25)*, processors *(3, 12, 15, 31)*, and consumers *(16, 20, 22)*. Fruits and vegetables are frequently in contact with soil, insects, animals, and humans during growing, harvesting *(32)*, and in the processing plant *(21)*. Thus, their surfaces are not free from natural contaminants, and by the time they reach the packing house, most fresh produce retain populations of 10^4 to 10^6 microorganisms/g *(6, 7)*. The level of sanitation and the microbiological load are of primary importance to the quality, shelf stability, and safety of fresh produce *(7, 8)*. Therefore, knowledge of the level of microflora on fruit or vegetable surfaces should help processors in implementing hazard analysis and critical control point (HACCP) plans and good manufacturing practices. However, estimation of the microbial load of a foodstuff is problematic, particularly where the surface of interest is uneven, as in the case of cantaloupe. Accordingly, the problem of obtaining a representative sample for examination is often difficult *(24)*.

Washing is one of the very first processing operations to which a fruit or vegetable is subjected. Washing can reduce microbial populations if done properly *(7)*. Washing usually consists of spraying with potable water or may involve use of disinfection by application of chlorine solutions. Chlorination of wash water has been reported to prevent microbial contamination in produce processing lines *(36)*. However, wide varieties of organic materials are subject to oxidation and chlorination reactions in aqueous chlorine solutions. Chlorination reactions are of particular concern in foods because of formation of potentially toxic chlororganic compounds *(37)*. In this study, the efficacy of hydrogen peroxide as an alternative to chlorine in reducing surface microflora of cantaloupe was investigated. While there are numerous reports in the literature on the use of chlorine as a sanitizer, information on the use of hydrogen peroxide on produce is limited.

Recovery of bacteria or other microorganisms from cantaloupe surfaces may not represent a true value due to the surface roughness of these fruits. The surface roughness favors microbial attachment and complicates detachment. Favero et al. *(11)* reviewed microbiological sampling methods for surfaces and summarized them into four basic methods: (i) the rinse, (ii) the swab rinse, (iii) agar contact, and (iv) direct surface agar plating. Methods 2 through 4 are not suitable for whole cantaloupe or other fruits with large surface area for the following reasons: (i) large amounts of media would be required; (ii) sampling would be time-con-

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† Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of similar nature not mentioned.

TABLE 1. *Comparison of extracting media for the bioluminescence ATP assay of cantaloupe surfaces*[a]

Extracting media	Log_{10} CFU/cm²[b]	Log_{10} ATP (fg/cm²)
Extralight	7.10 ± 0.12	5.14 ± 0.05
Chloroform-water (30% vol/vol)	7.00 ± 0.03	5.43 ± 0.47
Tris-EDTA[c]	7.18 ± 0.10	5.40 ± 0.04

[a] Values are means of five trials with duplicate determinations. All ATP extractions with the exception of Extralight involved boiling step.

[b] Determinations were performed immediately before ATP extraction and determination.

[c] 0.1 M Tris in 2 mM EDTA.

suming; and (iii) these methods may not give an accurate indication of total microbial counts on the surface. The rinse method involving the use of water would only be suitable for cantaloupe if adhering bacteria were all detached by the rinse and then enumerated in the rinse water. Since this is unlikely, new methods that can take into account size, shape, and surface properties of cantaloupe are needed.

Estimation of microbial numbers in foods by conventional microbiological techniques takes at least 2 to 3 days, and there is a need for faster methods that can give results in minutes. The bioluminescent ATP determination with firefly luciferase provides a sensitive indicator for the presence and growth of bacteria in biological material. The presence of bacteria in various media can be estimated using the bioluminescent ATP technique *(2, 5, 13, 14, 18, 34)*. Its use for rapid measurement of microbial cell numbers is based on the premise that all living things contain ATP and that the intracellular ATP levels have to be constant for cells to maintain normal physiological activities. Therefore, the level of ATP determined in a sample is proportional to the actual cell number *(30, 33)*. The assay does not require incubation and is therefore very rapid and sensitive. The purposes of this study were to examine a method that is suitable for extracting surface microbial ATP of cantaloupes and then use the method to monitor changes in the surface ATP level of cantaloupes stored at different temperatures. The correlation between log femtogram (fg) ATP and log CFU was investigated to determine the reliability of the bioluminescent ATP assay. Finally, the assay was used to compare the efficacy of hydrogen peroxide and chlorine solutions in reducing the surface microflora of cantaloupe.

MATERIALS AND METHODS

Preparation of sanitizers. Clorox, a commercial bleach containing 5.25% sodium hypochlorite (NaOCl; Clorox Company, Oakland, Calif.), was diluted in sterile water to provide the desired concentration (1,000 mg/liter) of chlorine in the wash solution. The pH was adjusted downward to 6.4 ± 0.1 by adding citric acid. Free chlorine in the solution was determined with a chlorine test kit (Hach Co., Ames, Iowa) that has been approved by the U.S. Environmental Protection Agency. Hydrogen peroxide was prepared from a 30% stock solution (Fisher Scientific, Suwannee, Ga.), which was diluted in sterile water to provide a concentration of 5% in the wash solution.

Cantaloupes. Cantaloupes purchased from a local supermarket and stored at room temperature (~20°C) or 4°C for 0, 24, 72, or 120 h were grouped (six per treatment) into the following categories: (i) unwashed; (ii) washed with tap water; and (iii) washed in sodium hypochlorite (1,000 mg/liter) or in hydrogen peroxide (5%) for 5 min. The concentrations of sodium hypochlorite and hydrogen peroxide were chosen based on the results of our previous laboratory work *(27)*. Chlorine- or hydrogen peroxide-treated samples were rinsed by dipping in sterile water and agitating with a glove-covered hand for 1 min, then air dried for 1 h at room temperature before sampling.

Sample preparation for microbiology. A sterilized stainless steel cork borer was used to cut randomly through the cantaloupe surface to produce rind plugs of 22 mm diameter with a surface area (πr^2) of 3.80 cm². A total of 152 rind plugs per cantaloupe

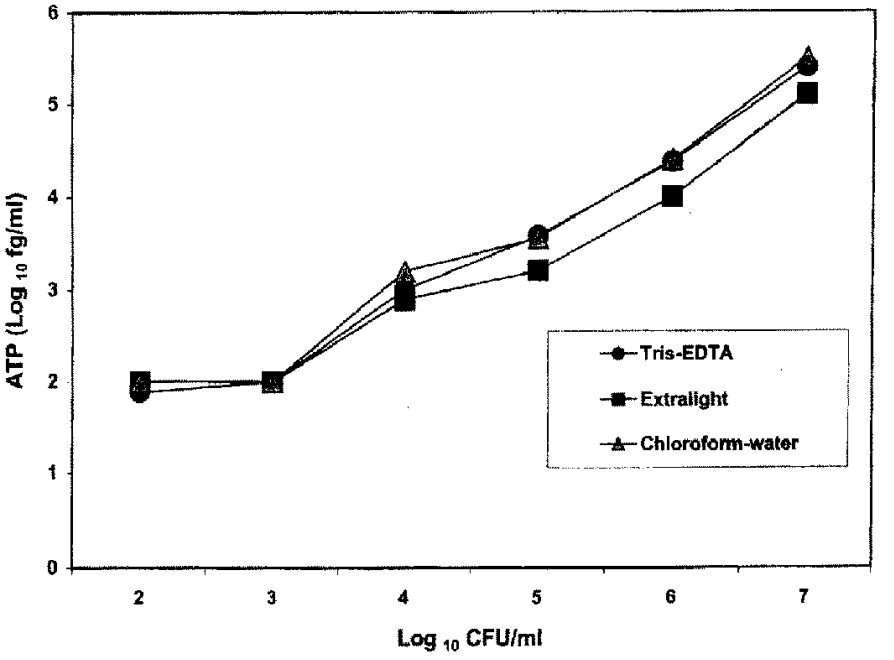

FIGURE 1. *Relationship between the plate count technique and the bioluminescence ATP assay of surface microflora of cantaloupes stored at 20°C for 24 h. Determinations of ATP were performed using 0.1 M Tris in 2 mM EDTA, 30% chloroform-water (vol/vol), and Extralight, a commercial detergent from Turner Design. Values are means of three determinations ± standard deviation. A correlation ($r^2 = 0.990$) between plate count numbers and ATP values was noted.*

TABLE 2. *Bioluminescence ATP estimation of the surface microflora of cantaloupe enumerated from different media*[a]

	Incubation temperature			
	25°C		35°C	
Media	Log_{10} CFU/cm^2	Log_{10} ATP (fg/cm^2)	Log_{10} CFU/cm^2	Log_{10} ATP (fg/cm^2)
PCA	6.61 ± 0.23	4.59 ± 0.18	7.59 ± 0.19	5.69 ± 0.15
PDA	3.30 ± 0.08	4.29 ± 0.15	1.16 ± 0.10	2.86 ± 0.14

[a] Cell enumeration and ATP determination were performed after incubation at 25°C for 48 h or at 35°C for 24 h. After cell enumeration, plates were flooded with 10 ml sterile dH_2O and then subjected to ATP extraction as stated in the "Materials and Methods." Values represent means of five trials with duplicate determinations ± standard deviation.

were obtained. The flesh adhering to the rind plugs was trimmed off using a sterilized stainless steel knife.

Microbiological analysis. Cantaloupe rind plugs (70) weighing approximately 25 g were blended (Waring commercial blender; Dynamic Corp., New Hartford, Conn.; the speed set at level 5, for 1 min) with 75 ml of 0.1% peptone water. Decimal dilutions of the sample were made with 0.1% peptone water, and aliquots (0.1 ml) were plated in duplicate on a range of media. Plate count agar (PCA; Difco, Detroit, Mich.) incubated at 25°C for 48 h or at 35°C for 24 h was used for the enumeration of total mesophilic aerobes. Potato dextrose agar (PDA; Difco), acidified with 10% tartaric acid to pH 3.5 and incubated at 25°C for 5 days, or at 35°C for 2 days, was used for yeast and mold enumeration. For the ATP determination, the plates were flooded with 10 ml sterile dH_2O, and the ATP was extracted as stated below.

Analytical reagent. ATP extraction buffers (0.1 M Tris-EDTA, pH 7.75, 30% [vol/vol] chloroform-water, and Extralight [Turner Design, Sunnyville, Calif.]) were used for the study. The Tris-EDTA was prepared by mixing 2 mM EDTA with 0.1 M Tris and adjusting the pH with 0.2 N HCl.

Bioluminescence ATP assay for surface microflora. Several ATP extractants (0.1 M Tris-EDTA, pH 7.75; 30% [vol/vol] chloroform-water; and Extralight [Turner design]) were studied to determine which solution would give the highest ATP yield. The method of Anhsen and Nilsson *(2),* as modified by Ukuku and Shelef *(33),* was used to determine level of bacterial ATP. Aliquots (1 ml) of samples taken for microbiological analysis plus 4 ml of one of the extracting solution listed above were mixed, followed by heating in boiling water for 5 min and cooling to room temperature in an ice bath. ATP extraction involving the Extralight was not subjected to the heating but was vortexed for 1 min. The ATP content was determined by the luciferin-luciferase reaction with an ATP bioluminescent assay kit (Sigma Chemical Co., St. Louis, Mo.). Bioluminescence ATP assays were performed using an ML 3000 microtiter plate luminometer (Dynatech Laboratories, Chantilly, Va.). The generated light signal was measured after a 16-s delay time and a 60-s integration time. Assays of standard amounts of ATP were used to calculate ATP levels, and values were expressed in log_{10} fg per cm^2 of cantaloupe surface. A control was run using 50 μl Tris-EDTA buffer in place of the ATP standard, and the control readings were subtracted as a measurement of background luminescence. Possible inhibition of the luciferase reaction by extracts or residues from the washing agents was corrected by adding known amounts of ATP standard into the reaction vial followed by addition of the luciferase enzyme. Based on the results of the ATP extraction study, the Tris-EDTA solution was chosen and used throughout for the ATP determination.

Data analysis. Five replicate trials for each treatment were conducted. Data from each treatment were subjected to the SAS software *(28)* for analysis of variance and for correlation of the two methods.

RESULTS AND DISCUSSION

ATP extraction study. Each of the ATP extraction methods gave similar results with cantaloupe surface mi-

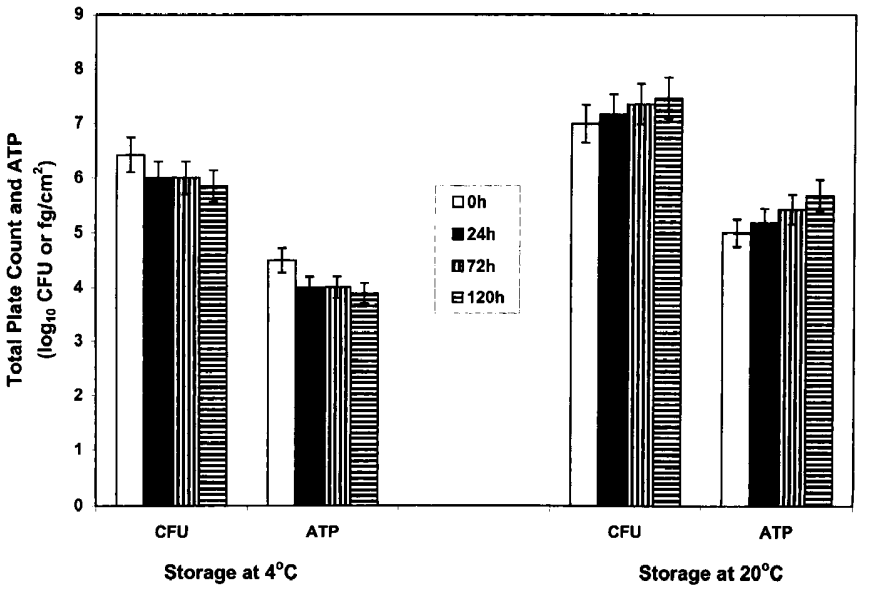

FIGURE 2. *Relationship between the plate count technique and the bioluminescence ATP assay of surface microflora of cantaloupes stored at 20 or 4°C for up to 120 h. Values are means of three determinations ± standard deviation.*

TABLE 3. *Estimation of viable count and ATP of surface microflora of cantaloupe after storage at 20 or 4°C for 24 h before treatment*[a]

Treatment	Storage temperature			
	20 ± 1°C		4 ± 1°C	
	Total plate count log CFU/cm^2	ATP log (fg/cm^2)	Total plate count log CFU/cm^2	ATP log (fg/cm^2)
Control	7.00 ± 0.09	4.98 ± 0.02	7.10 ± 0.10	4.95 ± 0.04
Water washed	7.30 ± 0.18	5.21 ± 0.06	7.18 ± 0.10	5.00 ± 0.07
Chlorine (1,000 ppm)	4.41 ± 0.15	2.83 ± 0.02 (2.50)	3.80 ± 0.16	2.48 ± 0.04 (2.68)
Hydrogen peroxide (5%)	4.12 ± 0.04	2.79 ± 0.04 (2.70)	4.49 ± 0.04	3.23 ± 0.09 (2.91)

[a] Values represent means of five trials with duplicate determinations ± standard deviation. Values in parentheses represent predicted ATP values if plate count numbers are set at 10^4 CFU. Calculation was based on the least-square formula ($Y = a + bX$), where predicted Y is ATP, (a) = the intercept, (b) = slope, and (X) = set value for minimum plate count number for surface microflora of cantaloupe.

croflora (Table 1). On all samples, the bioluminescence ATP determination gave an estimated ATP value of 5.30 log fg/cm^2 corresponding to an average total plate count of 7.1 log CFU/cm^2 total microflora on cantaloupe surfaces.

Sensitivity level of ATP extraction. The cantaloupe samples blended for microbiological assay of the surface microflora were diluted further to determine the sensitivity level of the bioluminescent ATP assay. The results suggest a linear relationship between cell numbers determined by the plate count method and the bioluminescence ATP value above 2.0 $\log_{10}$ fg/cm^2 (Fig. 1). Both the Tris-EDTA and chloroform-water appeared to extract slightly more ATP than Extralight. However, the standard deviation was higher with chloroform-water than with Tris-EDTA or the Extralight (Table 1). These results are in agreement with the report of Prioli and Brown *(26)*, who indicated better intracellular ATP extraction when boiling methods were utilized. All extractants used in the study were in agreement and correlated well with total microflora of surface cantaloupe above 3.0 $\log_{10}$ CFU/cm^2. Therefore, a sensitivity level of 3.0 $\log_{10}$ fg/cm^2 was established for this study. For the rest of the study, Tris-EDTA was used as the extractant to estimate bioluminescence ATP level of cantaloupe surface because of less variability, health reasons, and cost when compared to the chloroform or the Extralight.

With the bioluminescence assay, a 3.0 $\log_{10}$ fg/cm^2 surface microbial load on cantaloupes can be measured effectively. Below this threshold, the ATP determination showed a large variation and is therefore not reliable. This is in agreement with other reports *(19, 23)*. Karl *(17)* reported that vacuum filtration can be used to increase the sensitivity of the ATP assay to less than 10^4 CFU/ml. Griffiths *(14)* reported an approximate 70% agreement between the traditional plating techniques and the ATP assay rapid method. However, the bioluminescence ATP assay cannot distinguish ATP values from individual classes of microflora represented on the surface of cantaloupe. Yet, the assay provides an indication of the total microbial load, which may help establish a threshold for good manufacturing processes and HACCP guidelines. This technique can be utilized with modifications to assay any surface for microbial load or sanitary condition because of its versatility. Bioluminescent ATP techniques for estimating surface microbial load on cantaloupes can save the industry time by eliminating the required incubation applicable to the conventional methods.

Surface microflora of cantaloupe. To compare the bioluminescence ATP assay with the total plate count reliably, surface microflora of cantaloupes were enumerated using various media incubated at two growth temperatures (Table 2). Total microflora of cantaloupes enumerated using PCA incubated at 25°C for 48 h or at 35°C for 24 h were 6.61 and 7.59 $\log_{10}$ CFU/cm^2, respectively. Total ATP values corresponding to these organisms as determined by the bioluminescence ATP assay were 4.59 and 5.69 $\log_{10}$ fg/cm^2, respectively. The populations of yeast and mold of surface cantaloupe enumerated using PDA at 25 or 35°C were 3.30 or 1.16 $\log_{10}$ CFU/cm^2, respectively. The corresponding ATP values at 25 or 35°C were 4.29 and 2.86 $\log_{10}$ fg/cm^2, respectively. Total ATP values determined from the PDA were higher in contrast to those from PCA study, confirming a study that reported higher ATP/cell ratio in yeast than in bacteria *(2)*.

The bioluminescence ATP assay measures cellular components and metabolic by-products or actual growth; hence, it can be used to estimate bacterial numbers *(9, 10, 17)*. The relative light unit value of any bacterial bioluminescent is directly related to the ATP extracted and thus to the number of microbial cells from which it originated *(30)*. Bioluminescence ATP assays for estimation of viable counts on six cantaloupes analyzed ranged from 5.29 to 5.80 $\log_{10}$ fg/cm^2 ATP and correlated (r^2 = 0.938) with total plate counts of surface microflora of cantaloupe. However, Greeg *(13)* reported that ATP estimation of cell numbers by bioluminescence might not give good agreement with colony count on an agar plate for gram-positive cocci (staphylococci and streptococci). For example, the ATP count may show 10^5 CFU/ml, while the colony count shows 10^4 CFU/ml or less. The reason for such a discrepancy is that *Staphyloccus* and *Streptoccus* organisms grow in bunches and chains, respectively. Therefore, each CFU

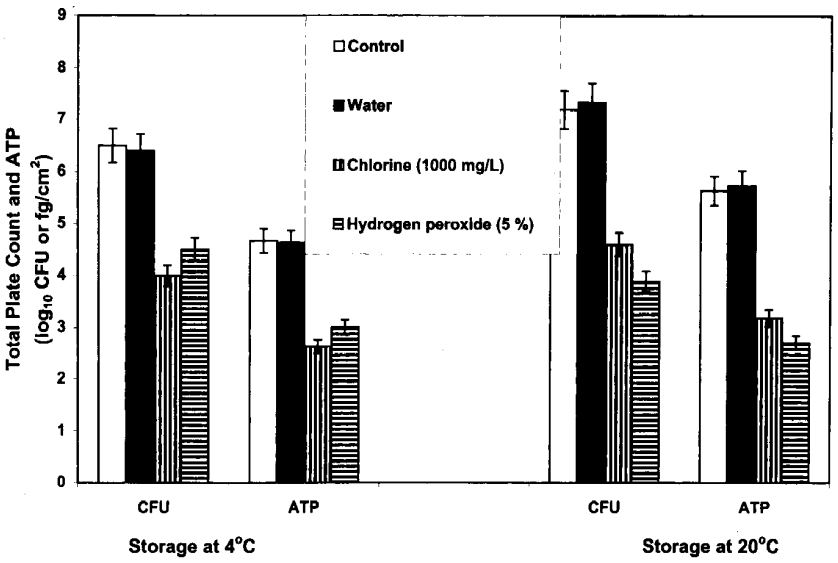

FIGURE 3. *Total plate count and bioluminescence ATP values of surface microflora of cantaloupes stored at 4 or 20°C and washed with water, chlorine (1,000 mg/liter), or hydrogen peroxide (50,000 mg/liter). Determination of ATP was performed using 0.1 M Tris in 2 mM EDTA. Values are means of three determinations ± standard deviation. A correlation (r^2 = 0.763) between plate count numbers and ATP values was noted.*

of these organisms may in fact represent 10 to 20 bacteria. This phenomenon may not apply in our study since the blending process utilized in this study may have dispersed any bacterial clumps on the cantaloupe surfaces before ATP extraction and determination.

Effect of storage temperature. Cell viability and total ATP content of surface microflora of cantaloupes stored at 20 or 4°C for 120 h were studied, and the results are shown in Figure 2. The population of surface microflora of cantaloupes stored at 20°C for 120 h and their corresponding ATP level increased slightly, probably due to an increase in yeast population. We have previously (Table 2) demonstrated that yeast has a higher ATP value in contrast to other surface microflora of cantaloupes. Total mesophilic aerobes on cantaloupes stored at 4°C declined to 6.0 log CFU/cm^2 after 24 h and remained the same throughout storage (Fig. 2). The same trend was observed using the bioluminescence ATP assay. The decline in total surface microflora and their corresponding ATP level could be attributed to the effect of cold temperature. Some of the aerobic mesophiles may have been injured by the cold storage temperature for 24 h, resulting in their death.

Effect of sanitizer on bioluminescent technique. Washing the cantaloupes with water did not cause changes in the total surface population count or the corresponding ATP value (Table 3 and Fig. 3). The chlorine treatment caused a 2.60 $\log_{10}$ CFU/cm^2 reduction in plate count and a 2.2 $\log_{10}$ reduction in ATP (fg/cm^2) of cantaloupes stored at 20°C for 24 h. The hydrogen peroxide treatment resulted in a 2.90 $\log_{10}$ CFU/cm^2 reduction of total plate counts and a 2.2 $\log_{10}$ ATP (fg/cm^2) reduction. The results at 120 h (data not shown) indicate the same trend as seen from the cantaloupes stored at 4 or 20°C for 24 h. The linear relationship between the plate count method and the bioluminescence ATP assays of cantaloupes treated with chlorine or hydrogen peroxide showed a similar correlation (r^2 = 0.999) between the two washing treatments in contrast to the unwashed melons (r^2 = 0.751) stored at 20°C for 120 h (Table 4). The slope (a) and intercept (b) of all the calculations, with the exception of the melons stored for 120 h without washing, showed the same trend, suggesting that this method can be used to predict ATP level on cantaloupe surfaces before and after washing treatment. Linear relationships between the plate count method and the biolu-

TABLE 4. *Comparison of linear regression and correlation coefficient of bioluminescence ATP assay and plate count of cantaloupe surfaces*

	Regression coefficients[a]					
	4°C			20°C		
Treatment	*a*	*b*	*r*	*a*	*b*	*r*
After purchase[b]	—	—	—	−1.585	1.126	0.995
Stored for 120 h	−8.927	2.167	0.980	3.280	0.283	0.751
Chlorine (1,000 mg/liter)[c]	−0.358	0.747	0.999	−0.812	0.826	0.999
Hydrogen peroxide (5%)[c]	0.274	0.658	0.999	−0.344	0.761	0.999

[a] Linear regression coefficients were calculated based on $Y = a + bx$ formula, where the slope (b), the y-intercept (a), and the correlation coefficient (r) for each plot are shown in the table.

[b] Performed immediately after purchase from the store.

[c] Treatments were applied after storage for 24 h at 4 or 20°C.

minescence ATP assays of cantaloupes determined immediately after purchase (r^2 = 0.995) and those stored at 4°C for 120 h (r^2 = 0.980) were similar, suggesting constant microbial population at this temperature. The lower linear correlation observed between the two methods on melons stored at 20°C for 120 h could be attributed to the possible growth of aerophilic organisms that grow in bunches or chains as suggested by Greeg *(13)*. It is also possible that the growth of yeast populations as observed in this study leads to the production of metabolites capable of producing quenching substances (compounds that withdraw or reduce light signal) since the melon surfaces were not washed before ATP determination (Fig. 3). Selan et al. *(29)* demonstrated the ability of chemical extractant to contribute to the quenching of bioluminescence. Valazquez and Feritag *(34)* observed a transition from enhancement to quenching in chemical sanitizers like alkaline foam, acid foam, commercial sodium hypochlorite, and D-limolene. They concluded that this effect was concentration dependent and that enhancement occurred at lower concentration.

CONCLUSION

The results of the bioluminescent ATP estimation of surface microbial load on cantaloupes correlated well with the plate count data. However, storage temperature and washing treatments affected the linear correlation between bioluminescent ATP estimation of surface microbial and total plate counts. This implies possible quenching activity of the bioluminescence process by the chemical treatments. Storage at 20°C for 120 h followed by washing treatments resulted in lower correlations compared to samples stored at 4°C for 24 h before washing. Results of this study suggest that hydrogen peroxide was similar in effectiveness to chlorine in reduction of surface bacterial population. Due to the reduced bacterial population, the total ATP values determined showed great variability and were below the minimum established threshold we reported.

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